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Effect of MarA-Like Proteins on Antibiotic Resistance and Virulence in *Yersinia pestis*▽

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MarA, an AraC/XylS transcriptional regulator in *Escherichia coli*, affects drug susceptibility and virulence. Two MarA-like proteins have been found in *Yersinia pestis*: MarA47 and MarA48. Deletion or overexpression of these proteins in the attenuated KIM 1001 Δ*pgm* strain led to a change in multidrug susceptibility (including susceptibility to clinically relevant drugs). Additionally, lung colonization by the *marA47* or *marA48* deletion mutant was decreased about 10-fold in a pneumonic plague mouse model. Complementation of the deletions by replacing the deleted genes on the chromosome restored wild-type characteristics. These findings show that two MarA homologs in *Y. pestis* affect antibiotic susceptibility and virulence.

Plague is a highly lethal disease caused by zoonotic *Yersinia pestis*. The different types of plague depend on the route of infection. Bites from infected fleas or animals result in bubonic plague or, in some cases, in primary septicemic plague. Inhalation of aerosolized *Y. pestis* causes primary pneumonic plague. Secondary septicemic plague and secondary pneumonic plague may also result from untreated bubonic plague (28, 38). Mortality rates in untreated patients range between 30 and 100%, with pneumonic plague being the most lethal (38). Even with prompt antibiotic treatment, mortality in patients with bubonic plague is 16%, and in patients with pneumonic plague, it is greater than 50% (28, 38). The last 15 years have seen an increase in the number of cases and epidemics of plague such that it is now considered a reemerging disease (10, 11, 38). Strains resistant to antibiotics favored in the treatment of plague have been identified (10). The absence of a reliable vaccine (23, 40), the need for antibiotics for treatment, and a history of use as a bioweapon (10, 28) make *Y. pestis* a worrying potential cause of another devastating event. The more we learn about the biology of this severe pathogen, the more effectively we can prevent or stop an epidemic.

The AraC/XylS family transcriptional regulator MarA was identified in *Escherichia coli* as a mediator of resistance to a variety of antimicrobial compounds (6, 13). This resistance is associated with its large regulon (2), genes of which are also found in the regulons of two other AraC/XylS members, SoxS and Rob (3, 26). The AraC/XylS family of transcriptional regulators is grouped together through the alignment of a two-helix-turn-helix DNA binding domain that is 99 amino acids in length (12). As well as a DNA binding domain, these proteins generally also contain a separate ligand binding region that is thought to be regulatory. For example, the C-terminal domain (non-DNA binding domain) of Rob is able to bind bile salts that cause it to be released from a sequestered inactive state (14, 32). MarA and SoxS, among others, form a subgroup of the AraC/XylS family with no ligand binding domain but are regulated by a transcriptional repressor (MarR) or transcriptional activator (SoxR), respectively. MarA orthologs (including SoxS and Rob) are found in many bacterial species, although the operon, *marORAB*, has been detected in only a few other members of the *Enterobacteriaceae*, e.g., *Salmonella*, *Shigella*, and *Klebsiella* spp. (verified using NCBI Genome [http://www.ncbi.nlm.nih.gov/]). In this operon, MarR binds to *marO*, repressing transcription of itself, *marA*, and *marB*. If this repression is relieved, as occurs in the presence of an inducer such as sodium salicylate, MarR can no longer bind to *marO*, and *marR*, *marA*, and *marB* are transcribed. MarA can also bind to *marO* and autoactivate transcription of the operon (1). In *E. coli*, MarA, SoxS, and Rob are involved in virulence, as demonstrated in a mouse model of ascending pyelonephritis (4).

A FASTA search of the *Y. pestis* and *Yersinia pseudotuberculosis* genomes revealed three potential chromosomal MarA orthologs. None of these was present in a *marORAB*-like operon. Here we report that two of these *marA*-like genes (*marA47* and *marA48*) are involved in antibiotic resistance and in virulence of *Y. pestis*. We used the attenuated *Y. pestis* strain KIM 1001 Δ*pgm*. This strain is an isogenic derivative of the KIM 10 strain (8, 37) and possesses all three virulence plasmids of *Y. pestis* (pCD1, pMT1, and pPCP1) but lacks the pigmentation (*pgm*) locus. The *pgm* locus carries two sets of genes: the high-pathogenicity-island (HPI), coding for the iron scavenging siderophore yersiniabactin, and the pigmentation segment, which includes the hemin storage (*hms*) locus necessary for colonization of the flea insect vector. Loss of this locus makes the KIM 1001 Δ*pgm* a biosafety level 2 organism (25, 42).

MATERIALS AND METHODS

Bacterial growth. The *Y. pestis* parent strain, KIM 1001 Δ*pgm* (37), was a gift from John Goguen (University of Massachusetts Medical School). For general growth, all *Y. pestis* strains were grown on tryptic blood agar plates (North East Laboratories) or in heart infusion (HI) broth (BD Difco). For other experiments (see below), sheep blood was obtained from North East Laboratories and tryptose blood agar base (TBAB) powder and brain heart infusion (BHI) broth and agar powder were obtained from BD Difco and made up according to the

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TABLE 1. Strains and plasmids

Strain or plasmid	Characteristics	Reference
Strains		
Y. pestis		
KIM1001 Δ*pgm* (WT)	Parental strain with *pgm* locus deleted, pCD1, pMT1, pPCP1	37
ILKIM1	WT but Δ*marA47*	This study
ILKIM9	WT but Δ*marA48*	This study
ILKIM11	ILKIM1 but Δ*marA48*	This study
ILKIM42	WT with pIL19.3	This study
ILKIM43	ILKIM1 with pIL19.3	This study
ILKIM45	ILKIM11 with pIL19.3	This study
ILKIM54	WT with pRU1	This study
ILKIM55	ILKIM1 with pRU1	This study
ILKIM56	ILKIM11 with pRU1	This study
ILKIM66	WT but Δ*lacZ*	This study
ILKIM84	ILKIM11 but *lacZ*::*kan*	This study
ILKIM88	WT but *lacZ*::*kan*	This study
ILKIM128	ILKIM9 but *lacZ*::*marA48*	This study
ILKIM131	ILKIM1 but *lacZ*::*marA47*	This study
ILKIM132	ILKIM11 but *lacZ*::*marA47*	This study
E. coli		
S17	Parental strain with λ*pir*	36
ILS17_1	S17 with pIL5i	This study
ILS17_4	S17 with pIL12.1	This study
ILS17_16	S17 with pIL41.6	This study
ILS17_21	S17 with pIL60.1	This study
ILS17_22	S17 with pIL64.1	This study
ILS17_MLF31	S17 with plasmid MLF31	This study
Plasmids		
pIL4.2	pGEM with *marA47* deletion fragment (pGEM kit)	This study
pIL10.1	pGEM with *marA48* deletion fragment (pGEM kit)	This study
pIL5i	pSR47s with *marA47* deletion fragment (NotI)	This study
pIL12.1	pSR47s with *marA48* deletion fragment (NotI)	This study
pIL19.3	pJP105 (*soxS* removed) with *marA48* (HindIII/BamHI)	This study
pIL41.6	MLF31 with kanamycin resistance gene from pKRP11	This study
pIL58.3	pGEM with *marA48* complementation fragment (pGEM kit)	This study
pIL60.1	MLF31 with *marA48* complementation fragment (SphI)	This study
pIL63.1	pGEM with *marA47* complementation fragment (pGEM kit)	This study
pIL64.1	MLF31 with *marA47* complementation fragment (SphI)	This study
MLF31	pCVD442 with *lacZ* deletion fragment	9
pJP105	*soxS* upstream of *lacZ* promoter	27
pKRP11	Kanamycin resistance cassette plasmid	30
pRU1	pJP105 (*soxS* removed) with *marA47* (HindIII/BamHI)	41
pSR47s	Suicide vector with kanamycin resistance and levansucrase gene *sacB*	22

manufacturer's instructions. All *E. coli* strains were grown on LB agar (BD Difco LB powder plus 5 g/liter NaCl) or LB broth (BD Difco LB powder plus 5 g/liter NaCl). To maintain plasmids in bacteria, the appropriate antibiotics to which plasmids specified resistance were added to the media before growth of plasmid-bearing strains.

Genetic manipulation. All strains and plasmids are listed in Table 1; all primers used for strain construction are listed in Table 2. Chromosomal sequences for *Y. pestis* KIM and CO92 were obtained from the Wellcome Trust Sanger Center sequencing projects website (http://www.sanger.ac.uk/Projects/Y_pestis/) and the NCBI genome website (http://www.ncbi.nlm.nih.gov/sites/entrez?db=Genome&itool=toolbar), respectively. Genomic DNA was prepared from cultures grown overnight in HI broth using the Wizard Genomic DNA isolation kit (Promega). Coding regions for chromosomal genes *marA47* (coordinates 25192750 to 2520168) and *marA48* (coordinates 479842 to 480708) were deleted following the method of Heckman and Pease (16) using the suicide vector pSR47s (22). Primers were designed to anneal ~500 bp upstream and downstream of *marA47* (primers IL-5 and IL-6) and *marA48* (primers IL-29 and IL-32). Primers were also designed such that half annealed with a ~20-bp region just upstream of the start codon and the other half annealed to ~20 bp just downstream of the stop codon (primer IL-4 for *marA47* and primer IL-31 for *marA48*). The reverse complement to these primers was also created (IL-7 for *marA47* and primer IL-30 for *marA48*). PCR, using KIM 1001 Δ*pgm* genomic DNA as a template, was used to create ~500-bp fragments with IL-4/IL-5 and IL-6/IL-7 for *marA47* and IL-29/IL-30 and IL-31/IL-32 for *marA48*. Using the overlapping sequences from primers IL-4, IL-7, IL-31, and IL-30, a third PCR was used to splice fragment IL-4/5 together with fragment IL-6/7 and fragment IL-29/31 together with fragment IL-31/32, thus creating ~1,000-bp fragments of the ~500-bp regions flanking *marA47* and *marA48*. The ~1,000-bp fragment was subcloned into pGEM using the pGEM-T-easy vector kit (Promega), creating plasmids pIL4.2 and pIL10.1 for *marA47* and *marA48*, respectively. The fragment was then excised with NotI (Invitrogen) and cloned into the NotI-digested suicide vector pSR47s, which carries a kanamycin resistance gene and the levansucrase gene, *sacB*, using *E. coli* S17 λ*pir* (36), creating plasmids pIL5i in *E. coli* strain ILS17_1 (*marA47*) and pIL12.1 in *E. coli* strain ILS17_4 (*marA48*). Bacterial conjugation was performed with wild-type (WT) *Y. pestis* KIM 1001 Δ*pgm* and *E. coli* ILS17_1 or *E. coli* ILS17_4. *Y. pestis* colonies containing recombinant pSR47s were selected on LB agar plates supplemented with 50 μg/ml kanamycin and 1 μg/ml irgasan (selective for *Yersinia*) (20). After colony purification, single colonies were grown in HI broth and serially diluted onto tryptose blood agar plates supplemented with 10% sucrose and 5% sheep blood or with 50 μg/ml kanamycin and 5% sheep blood to select for strains resistant to sucrose (having lost the pSR47s *sacB* gene) or sensitive to kanamycin (having lost the pSR47s

TABLE 2. Primers

Primer	Sequence (5′ → 3′)[a]	Coordinates[b]
IL-4	GTATATATATAAAGAATAATAAATAACGCCCGATTAGCATCATTTAGC	2519252–2519274/2520166–2520190
IL-5	GCGGGATCCCCGATGCTGATCAACAAACAG (BamHI)	2133050–2133070
IL-6	ATAAGAATGCGGCCGCCGGTGTTACAGGCCTGAATTTCATG (NotI)	2518763–2518785
IL6SphI	ACATGCATGCCGGTGTTACAGGCCTGAATTTCATG (SphI)	2518763–2518785
IL-7	GCTAAATGATGCTAATCGGGCGTTATTTATTATTCTTTATATATATAC	2133539–2133563/2134455–2134477
IL-29	ATAAGAATGCGGCCGCGGTATCACGCATATTATTAGC (NotI)	479336–479356
IL-30	GCTGTTTTACGAGGAAGTTTTCGCTGTAATTCATCAAGTGCTGG	4173000–4173020/417888–4713910
IL-31	CCAGCACTTGATGAATTACAGCGAAAACTTCCTCGTAAAACAGC	479819–479841/480709–480729
IL-32	GCGGGATCCCACTTAGCTCAATCGGCCCAAC (BamHI)	4172522–4172543
IL-69	GGCATAAGCTTATGGATCAAGCCAGTATCATTCGTG (HindIII)	4173021–4173045
IL-70	GCCGGATCCTTAACGGCGAATTGGAATGAAATAG (BamHI)	479842–479866

[a] Restriction enzyme sites are underlined, and restriction enzymes are in parentheses.
[b] Gene coordinates are from *Y. pestis* CO92 (Wellcome Trust Sanger Center sequencing projects website, http://www.sanger.ac.uk/Projects/Y_pestis/).

kanamycin resistance gene) and therefore deleted of the gene of interest. PCR amplification, and sequencing confirmed the deletion. The resulting strains, ILKIM1, ILKIM9, and ILKIM11, lack *marA47*, *marA48*, and *mar47* and *marA48*, respectively. ILKIM11 was created by deleting *marA48* from ILKIM1.

To create complemented strains, primer pairs IL5SphI/IL-6 and IL-29/IL-32 were used to amplify *marA47* and *marA48* sequences from KIM 1001 Δ*pgm* genomic DNA. These PCR products were subcloned into pGEM using the pGEM-T-easy kit (Promega), creating plasmids pIL63.1 and pIL58.3 for *marA47* and *marA48*, respectively. *marA47* and *marA48* were excised from these plasmids using SphI and cloned into the MLF31 plasmid (9) using S17 λ*pir*, such that each gene was flanked by 500-bp regions carrying for the *Y. pestis lacZ* gene, creating plasmid pIL64.1 and *E. coli* strain ILS17_22 for *marA47* and plasmid pIL60.1 and *E. coli* strain ILS17_21 for *marA48*. *E. coli* ILS17_22 was conjugated with ILKIM1 and ILKIM11, and *E. coli* ILS17_21 was conjugated with ILKIM9. Recombination events were selected for as described for creation of the deletion strains, with the following differences: ampicillin was used to select for recombinant strains, as MLF31 codes for ampicillin resistance rather than kanamycin resistance, and 40 μg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was added to the TBAB agar supplemented with 10% sucrose and 1% sheep blood. Thus, strains ILKIM128 (Δ*marA48 lacZ*::*marA48*), ILKIM131 (Δ*marA47 lacZ*::*marA47*), and ILKIM132 (Δ*marA47* Δ*marA48 lacZ*::*marA47*) were created. To create ILKIM66, a knockout mutant of the *lacZ* gene in the WT strain, *E. coli* S17 λ*pir* was transformed with the MLF31 plasmid to create the strain *E. coli* ILS17_MLF31, which was conjugated with the KIM 1001 Δ*pgm* strain. Bacteria lacking *lacZ* were selected in the same way as described for the complementation strains. To create strains with a kanamycin resistance cassette inserted into the *lacZ* gene on the chromosome, the kanamycin resistance cassette was removed from plasmid pKRP11 (30) using SphI and ligated into the SphI-digested MLF31 plasmid to produce pIL41.6 in *E. coli* strain ILS17_16. *E. coli* ILS17_16 was conjugated with the WT *Y. pestis* strain and *Y. pestis* strain ILKIM11 and selected as described above but using ampicillin to select for the first recombination event and kanamycin to select for and verify the second recombination event, to create *Y. pestis* ILKIM88 and ILKIM84, respectively.

To create the MarA48 overexpression strain, plasmid pJP105 (27), a gift from Bruce Demple (Harvard School of Public Health, Boston), which contains the *lacZ* promoter upstream of the *E. coli soxS* gene and an ampicillin resistance gene as a selectable marker, was used. The *soxS* gene was removed using BamHI and HindIII. Primers IL-69 and IL-70 were used to amplify the *marA48* gene from KIM 1001 Δ*pgm* genomic DNA, and the PCR product was digested with BamHI and HindIII before being ligated into the digested pJP105 plasmid. The ligation reaction was transformed into DH5α cells. The resulting plasmid, pIL19.3, was verified by sequencing and transformed into the *Y. pestis* WT, ILKIM9, and ILKIM11 strains to create strains ILKIM42 to -44. The *Y. pestis* WT, ILKIM1, and ILKIM11 strains were transformed with pRU1, which contains pJP105 with *marA47* in the place of *soxS*, to create strains ILKIM54 to -56.

Conventional molecular biology techniques were used for all other genetic manipulations. Transformation of any bacterial strains with plasmid DNA was performed using electroporation of calcium-competent bacteria using the Bio-Rad Micropulser. All primers were synthesized by Integrated DNA Technologies (Coralville, IA), and all DNA sequencing was performed by the Tufts University Medical School Core Facility.

Growth curves and coculture studies. The *Y. pestis* WT strain, isogenic strains with different gene deletions ILKIM1 (Δ*marA47*), ILKIM9 (Δ*marA48*), and ILKIM11 (Δ*marA47* Δ*marA48*), and the complemented strains ILKIM128 (Δ*marA48 lacZ*::*marA48*), ILKIM131 (Δ*marA47 lacZ*::*marA47*), and ILKIM132 (Δ*marA47* Δ*marA48 lacZ*::*marA47*) were grown in HI broth overnight at 26°C. In the morning, the cultures were diluted 10-fold and grown for 3 h at 26°C. The optical density at a wavelength of 600 nm (OD_{600}) was measured, and all cultures were diluted to an OD_{600} of 0.1. Using a 96-well plate, 100 μl of culture was placed into each well, using a minimum of three wells per strain. A Perkin-Elmer Victor^3 1420 multilabel reader measured the growth rates at 26°C. The 96-well plate was shaken every 5 min, and readings were taken every 20 min over a 20-h period.

Growth curves were also determined to test the effect of IPTG (isopropyl-β-D-thiogalactopyranoside) on growth rates. Cultures of the WT strain and strains ILKIM42 (WT plus pIL19.1), ILKM43 (ILKIM9 plus pIL19.1), ILKIM44 (ILKIM11 plus pIL19.1), ILKIM54 (WT plus pRU1), ILKIM55 (ILKIM1 plus pRU1), ILKIM56 (ILKIM11 plus pRU1), ILKIM1, ILKIM9, and ILKIM11 were grown overnight at 26°C in HI. In the morning, the cultures were diluted to an OD_{600} of 0.1 and allowed to grow for 3 h at 26°C. The OD_{600} was measured, and all cultures were again diluted to an OD_{600} of 0.1 before two tubes of each culture were prepared, one in HI and the other in HI supplemented with 1 mM IPTG. Growth curves were measured as described above using the Victor^3 1420 multilabel reader and a 96-well plate.

Coculture studies were performed using the following pairs of strains beginning at an OD_{600} of 0.01: ILKIM88 and WT, ILKIM88 and ILKIM1, ILKIM88 and ILKIM9, ILKIM88 and ILKIM11, and ILKIM84 and WT. After growth at 26°C for 48 h, cultures were plated on TBAB agar supplemented with 40 μg/ml X-Gal. The numbers of blue and white colonies (CFU) were counted.

Antibiotic susceptibility testing. Bacterial strains were grown overnight in HI broth at 26°C and diluted 10-fold before a further 3 h of growth at 26°C. The OD_{600} of each culture was then measured, and the cultures were diluted to an OD_{600} of 0.05 and uniformly spread on BHI or TBAB agar plates on which Etest strips (AB Biodisk, Solna, Sweden) were placed. Plates were incubated at 26°C for 2 days, after which the MICs were recorded. To test antibiotic susceptibility during MarA47 or MarA48 overexpression, *Y. pestis* strains with overexpression plasmids (ILKIM43, -44, and -54 to -56) were grown as described above in HI broth supplemented with the selecting antibiotic, ampicillin, at 100 μg/ml. After the 3-h growth period, cultures were diluted 2-fold and divided in half, and 1 mM IPTG was added to one half of each culture before growth for a further 3 h at 26°C. Cultures grown with IPTG were plated on agar containing 1 mM IPTG. MICs were determined as described above using Etest strips.

Mouse lung infection. *Y. pestis* strains were grown overnight at 26°C, diluted 10-fold, and grown for 3 h at 26°C. The cultures were then diluted in phosphate-buffered saline (PBS) to give 5×10^6 CFU/ml. Seven- to 8-week-old female BALB/c mice were anesthetized with isofluorane and 40 μl (2×10^5 CFU) of bacterial suspension slowly delivered into both nares. Mice were allowed to recover and then left for 5 days before they were euthanized and the lungs removed. The tissue was homogenized in 1 ml of 15% glycerol in PBS, serially diluted in PBS, and plated on tryptose blood agar base supplemented with 1 μg/ml irgasan (20). Tubes containing the 1 ml of 15% glycerol in PBS were weighed before and after the addition of the lung tissue in order to determine CFU/g lung. The limit of detection was log 2.6 CFU/g lung. Results of at least three experiments were determined. After logarithmic transformation of the data, significant differences between WT and mutant strains were determined using the Kruskal-Wallis test with Dunn's posttest and the Mann-Whitney test

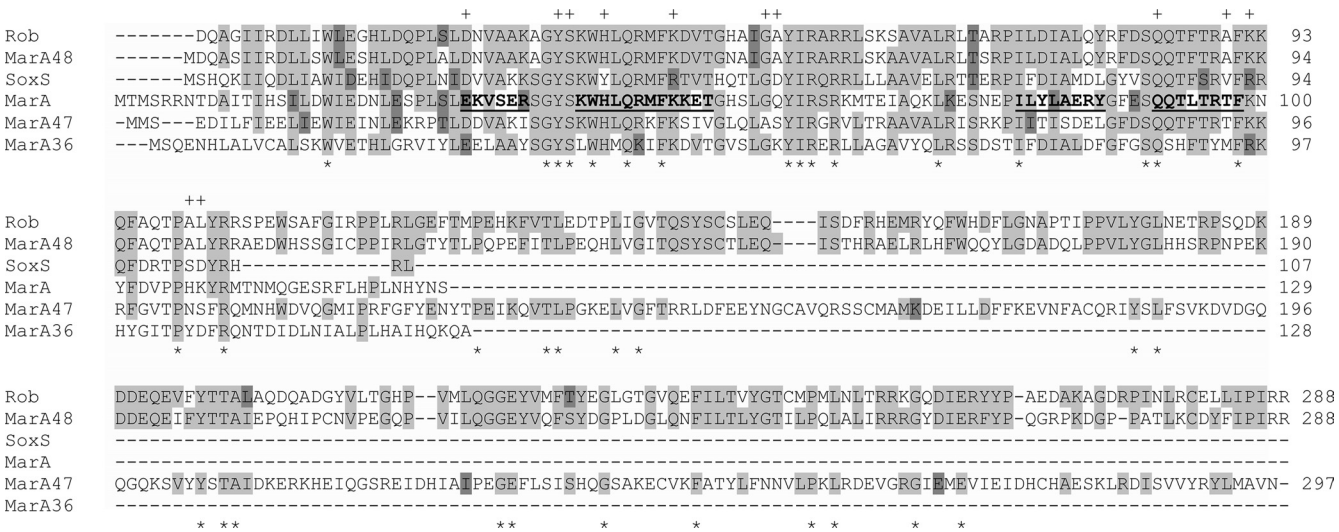

FIG. 1. Alignment of MarA, SoxS, and Rob from *E. coli* with *Y. pestis* homologs MarA47, MarA48, and MarA36. Asterisks denote complete identity, light gray shading shows the presence of three or more identical residues, and dark gray shading shows residues with chemically similar properties. Residues in bold and underlined denote the helices of the two helix-turn-helix motifs of MarA. + denotes residues in MarA that contact the DNA phosphate backbone, as described by Rhee et al. (31).

using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA). All animal procedures were performed using protocols approved by the Tufts Department of Animal Medicine and the Institutional Animal Care and Use Committee.

RESULTS AND DISCUSSION

***E. coli* MarA-like proteins in *Y. pestis*.** A BLAST search of the *Yersinia pestis* CO92 chromosome (using the Sanger Center website, http://www.sanger.ac.uk/cgi-bin/blast/submitblast/y_pestis) revealed four chromosomal and two plasmid-specified MarA-like proteins, as described previously (41). We performed a further search using FASTA, which aligns full-length proteins rather than domains. This search identified three of the four original chromosomal homologs, namely, MarA36, MarA47, and MarA48. A ClustalW (39) alignment of these three chromosomal proteins was made with the *E. coli* proteins MarA, Rob, and SoxS (Fig. 1). The residues of the helix-turn-helix motif that contact the DNA helix are conserved with those of the *E. coli* proteins (31). MarA and SoxS are unusual in the AraC/XylS family in having no ligand binding domain; regulation of their expression occurs through the repressing MarR and activating MarA or SoxR proteins. Unlike that of MarA and SoxS, the binding of Rob to its target DNA is thought to be regulated through a ligand binding to its C terminus (3, 32) that releases it from a sequestered state and prevents degradation during inactivity (14). From the alignment (Fig. 1), we see that while the C terminus of MarA47 has some identity with the last 166 residues of Rob (21%), there is substantially more identity between the C termini of MarA48 and Rob (54%). In these alignments the C terminus was defined as starting with the residue that follows the last *E. coli* MarA residue. It is possible that the C-terminal domains of MarA47 and MarA48 might also provide a regulatory mechanism for these proteins instead of, or in addition to, a regulatory protein such as MarR or SoxR. Finally, MarA36 is the closest in length to MarA but presents the least identity.

Antibiotic susceptibility testing. Streptomycin and gentamicin (aminoglycosides), tetracycline (in a class named after itself), and chloramphenicol (a phenicole) are favored antibiotics for treatment of plague patients (10). All four bind the ribosome and inhibit protein synthesis. The fluoroquinolone ciprofloxacin is an important alternative for plague treatment that acts by inhibiting DNA gyrase. Tetracycline and sulfonamide-containing drugs such as trimethoprim-sulfamethoxazole are used prophylactically (10). Trimethoprim is a dihydrofolate reductase inhibitor, and sulfamethoxazole is an inhibitor of dihydropteroate synthetase, an enzyme involved in DNA synthesis. For aerosolized plague used as a bioweapon, all four antibiotics are recommended, as well as the tetracycline doxycycline (10).

With the antibiotics used for plague treatment and prophylaxis in mind, the antibiotic susceptibilities of strains with altered expression of *marA47* and *marA48* were studied using the *Y. pestis* WT strain KIM1001 Δ*pgm* and isogenic deletion mutants, their complemented strains, and strains overexpressing each gene. Previous studies showed no effect on antibiotic susceptibility during overexpression of the MarA36 protein in *Y. pestis* (41), and our Etest assays demonstrated that deletion of *marA36* alone, as a double mutation with *marA48*, or as a triple mutation with *marA47* and *marA48* caused no change in antibiotic susceptibility compared with the *Y. pestis* WT, Δ*marA47*, Δ*marA48*, or Δ*marA47* Δ*marA48* strains, respectively (data not shown). *marA36* was therefore not studied further.

Antibiotic susceptibilities were determined for the *Y. pestis* KIM1001 Δ*pgm* WT and the isogenic deletion mutants ILKIM1 (Δ*marA47*), ILKIM9 (Δ*marA48*), and ILKIM11 (Δ*marA47* Δ*marA48*) on both BHI and TBAB agar using the Etest assay. Patterns of susceptibility were similar on both types of agar, though MICs were different for some antibiotics. Figure 2 shows a representative experiment with TBAB agar; at least three replicates for each strain were performed in total.

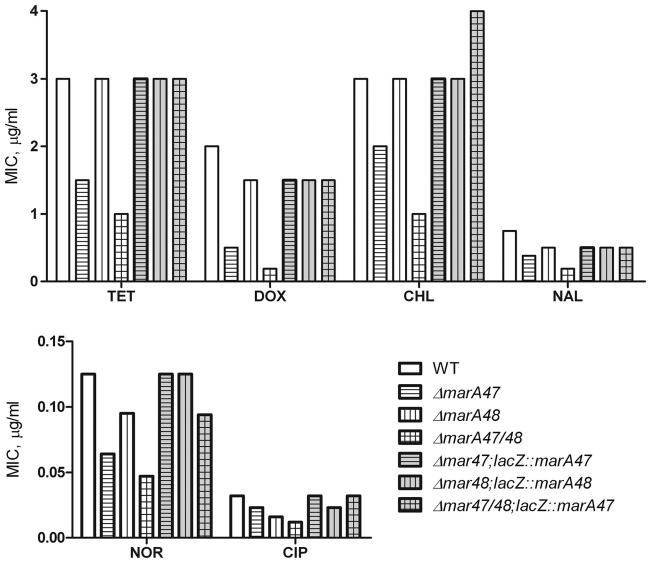

FIG. 2. Drug susceptibilities of wild-type KIM 1001 Δ*pgm* and deletion mutants. The graph shows results of a representative experiment from at least three experiments. WT, deletion, and complemented deletion strains (ILKIM1 [Δ*marA47*], ILKM9 [Δ*marA47*], ILKIM11 [Δ*marA47* Δ*marA48*], ILKIM128 [Δ*marA48 lacZ*::*marA48*], ILKIM131 [Δ*marA47 lacZ*::*marA47*], and ILKIM132 [Δ*marA47* Δ*marA48 lacZ*::*marA47*]) were grown at 26°C in HI broth and plated at an OD_{600} of 0.05. Etest strips were laid down and MICs recorded after incubation at 26°C for 2 days. TET, tetracycline; DOX, doxycycline; CHL, chloramphenicol; NAL, nalidixic acid; NOR, norfloxacin; CIP, ciprofloxacin.

MICs in individual experiments varied slightly but always followed the same trend. The loss of *marA47* led to a 25 to 60% increase in susceptibility to tetracycline, doxycycline, chloramphenicol, nalidixic acid (a quinolone), and norfloxacin and ciprofloxacin (both fluoroquinolones) compared to that of the WT KIM1001 Δ*pgm*. The deletion of *marA48* alone caused no increase in susceptibility except for the fluoroquinolone norfloxacin, where there was a consistent increase in susceptibility of 25 to 50%. The deletion of *marA47* together with *marA48* (ILKIM11) increased susceptibility by 35 to 75%, and the MIC was always less than the MIC for the Δ*marA47* (ILKIM1) strain. This finding suggests that MarA47 and MarA48 have synergistic functions and that MarA47 potentially compensates for the loss of *marA48* in the Δ*marA48* single mutant. None of the mutants showed a difference in comparison with the WT strain when tested against the sulfonamide-containing trimethoprim-sulfamethoxazole or the aminoglycosides gentamicin and streptomycin.

Though the increases in antibiotic susceptibility are small, there are several reports showing that adaptation to small increases in antibiotic concentration allow bacteria to develop clinically significant resistance to antibiotics. Mutations in the *marRAB* operon have been found in clinical isolates (17, 21, 24).

To ensure that the deleted genes could be complemented, the gene of interest, including a ~500-bp region upstream of the start codon (to accommodate the promoter), was inserted into the *lacZ* gene of the deletion strains, creating strains *Y. pestis* ILKIM128 (Δ*marA48 lacZ*::*marA48*), ILKIM131 (Δ*marA47 lacZ*::*marA47*), and ILKIM132 (Δ*marA47* Δ*marA48 lacZ*::*marA47*). Loss of the *lacZ* gene alone (strain ILKIM66) did not cause a change in susceptibility (results not shown). *marA47* was able to complement the antibiotic susceptibility phenotype in both the *marA47* single deletion (ILKIM131) and the Δ*marA47* Δ*marA48* (ILKIM132) strains (Fig. 2). *marA47* was consistently unable to completely complement for the increased susceptibility against the fluoroquinolone norfloxacin in the double deletion mutant, while *marA48* was able to complement its own deletion against this antibiotic. These results support the deletion phenotype, suggesting that MarA48 is involved in norfloxacin susceptibility (ILKIM128).

To verify that the difference in MICs was not due to differences in growth rate between the WT and isogenic deletion mutants, growth curve and coculture experiments were performed. No difference was seen in doubling times or the point at which the stationary phase was reached during the growth curves, nor was a difference seen when the WT strain was cocultured together with any of the mutant strains over a period of 48 h (data not shown).

For overexpression studies, WT and isogenic deletion strains were transformed with plasmids harboring the *Y. pestis marA* homologs under the control of a *lac* promoter. The induction of MarA47 expression in *Y. pestis* KIM1001 Δ*pgm* with IPTG from the pRU1 plasmid in the WT (ILKIM54), Δ*marA47* (ILKIM55), and Δ*marA47* Δ*marA48* (ILKIM56) strains more than compensated for its loss either alone or in combination with the *marA48* deletion and decreased the drug susceptibility by 4- to 10-fold for all the antibiotics tested, with the exception of streptomycin (data not shown), where there was no effect (Fig. 3A), supporting results reported previously with the *Y. pestis* EV76-51F strain where overexpression of MarA47 from pRU1 decreased antibiotic susceptibility (41). The results for the other aminoglycoside, gentamicin, were variable, with a 2-fold decrease in susceptibility only with overexpression of MarA47 in the WT strain (results not shown). The induction of MarA48 expression with IPTG, through the plasmid pIL19.3 in the WT (ILKIM42), Δ*marA47* (ILKIM43), and Δ*marA47* Δ*marA48* (ILKIM44) strains, was able to consistently decrease antibiotic susceptibility to the fluoroquinolone norfloxacin and the aminoglycoside gentamicin by 3- and 2-fold, respectively (Fig. 3B). No differences were seen in susceptibility to tetracycline, doxycycline, streptomycin, or nalidixic acid from induced MarA48 (data not shown). In agreement with the deletion studies, these finding show that while MarA47 can compensate for the effects of the loss of MarA48 and itself, MarA48 is unable to cover for the increased susceptibility associated with the loss of *marA47*, except for norfloxacin and gentamicin. The greater susceptibility seen with IPTG induction of pRU1 or pIL19.3 is linked to the copy number of 15 to 20 of the plasmids which are derivatives of pBR322 (27; Qiagen plasmid resource center). The susceptibility of untransformed strains was not affected by the presence of IPTG (data not shown). We also tested the effect of IPTG on growth rates to ensure that the presence of the compound did not confer a growth advantage. Growth rates for the untransformed strains (WT, ILKIM1, ILKIM9, and ILKIM11) and for transformed strains (ILKIM42, -43, and -54 to -56) were identical in the presence or absence of IPTG, with the exception of strains transformed with pRU1 (ILKIM54 to -56), where after the initial 2.5 h of growth, the presence of IPTG decreased the

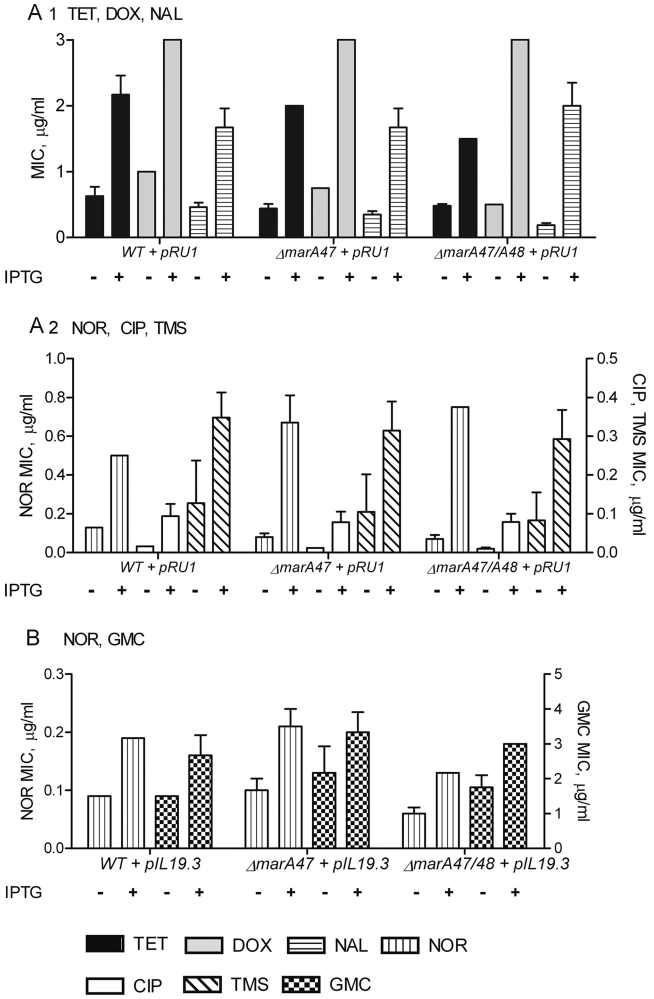

FIG. 3. Antibiotic susceptibilities of *Y. pestis* KIM 1001 Δ*pgm* strains overexpressing MarA47 or MarA48. *marA47* and *marA48* were cloned into the expression vector pJP105 to create plasmids pRU1 and pIL19.3. In the presence of IPTG, expression of MarA47 or MarA48 is induced. (A) pRU1 was transformed into the WT, Δ*marA47*, and Δ*marA47* Δ*marA48* strains, creating strains ILKIM54 to -56 and the MICs resulting from Etests of these strains in the absence and presence of IPTG are shown. (B) pIL19.3 was transformed into the WT, Δ*marA48*, and Δ*marA47* Δ*marA48* strains, creating strains ILKIM42 and -43, and the MICs resulting from Etests of these strains in the absence and presence of IPTG are shown. All strains were grown overnight in the presence of the selecting antibiotic, ampicillin. After a 10-fold dilution, all cultures were grown for a further 3 h with ampicillin, at which point the cultures were diluted 2-fold and split in half, 1 mM IPTG added to half the cultures, and all grown for a further 3 h. At this point the OD_{600} was measured. Cultures were plated on TBAB agar with or without 1 mM IPTG at an OD_{600} of 0.05. Etest strips were used to determine the MICs. Each bar shows the average from three experiments with standard deviations. Where no standard deviation bar is shown, there was no difference among experiments. TET, tetracycline; DOX, doxycycline; NAL, nalidixic acid; NOR, norfloxacin; CIP, ciprofloxacin; GMC, gentamicin.

growth rate about 2-fold (data not shown). This could be because a buildup of MarA47 protein was detrimental to growth. Thus, IPTG does not confer any growth advantage to the strains that would result in a higher MIC.

The differences in the MICs seen between the same strains described in Fig. 2 and 3, and which are particularly marked for tetracycline and doxycycline MICs, were caused by the use of TBAB agar for the experiments shown in Fig. 2 and BHI agar for the experiments shown in Fig. 3.

Taken together, these deletion and overexpression studies suggest that *Y. pestis* MarA47 has the greater role in susceptibility to antibiotics, including those used to treat plague, and that *Y. pestis* MarA48 may function to enhance the role of MarA47. In *E. coli*, deleting the *marORAB* operon has only a small effect on antibiotic susceptibility; overexpression of the operon, however, causes a more marked decrease in antibiotic susceptibility (1, 41). Inducing expression of MarA47 and MarA48 causes a decrease in antibiotic susceptibility that is larger than the increase in susceptibility caused by their deletion. This is particularly true for MarA47 and susceptibility to trimethoprim/sulfamethoxazole, and for MarA48-associated susceptibility to gentamicin, where a change in susceptibility was seen during induced expression but not when the genes were deleted. Like in *E. coli*, the *marA* homologs in *Y. pestis* are presumably in an inactive/repressed state under laboratory conditions and require environmental cues to be fully expressed and activated. Once activated, MarA47 and MarA48 up- or downregulate proteins involved with resistance and permeability, such as efflux proteins, outer membrane porins, and cell membrane/wall structural proteins. It has been reported that induced expression of MarA47 produces multidrug resistance through increased expression of AcrAB homologs in *Y. pestis* (41). Unlike the case for MarA in *E. coli*, however, overexpression of MarA47 and MarA48 in *Y. pestis* decreased susceptibility to trimethoprim-sulfamethoxazole and gentamicin, respectively.

Mouse lung infections. In the early stages of pneumonic plague infection, the bacterial load in the lungs increases dramatically and bacteria disseminate from there to cause a systemic infection (18). Thus, the ability to colonize the lungs is associated with the virulence of *Y. pestis*. We infected 7- to 8-week-old female BALB/c mice intranasally with 2×10^5 CFU of the *Y. pestis* KIM 1001 Δ*pgm* WT strain; the isogenic deletion strains ILKIM66 (Δ*lacZ*), ILKIM1(Δ*marA47*), ILKIM9 (Δ*marA48*), and ILKIM11 (Δ*marA47* Δ*marA48*); and the isogenic complementation strains ILKIM131 (Δ*marA47 lacZ*::*marA47*) and ILKIM128 (Δ*marA48 lacZ*::*marA48*) strains. After 5 days, mice were euthanized, and the lungs were homogenized, serially diluted, and plated on tryptose blood agar base (BD Difco) containing 1 μg/ml irgasan (Fig. 4). The loss of the *lacZ* gene did not have an effect on the virulence of the WT strain, and the presence of 1 μg/ml irgasan did not affect the numbers of CFU for the WT strain or any of the mutant strains (results not shown). Mutant strains with the single deletions of *marA47* and *marA48* and the double deletions of *marA47* and *marA48* were attenuated, showing a ~10-fold decrease in lung colonization relative to the WT. The differences were significant using the Mann-Whitney test. The Kruskal-Wallis nonparametric one-way analysis of variance (ANOVA) with Dunn's posttest also showed that the single Δ*marA47* deletion strain is significantly different from the WT. The medians of data for the WT, Δ*marA47* (ILKIM1), and complemented Δ*marA47* (ILKIM131) strains show that the attenuation is due to the loss of *marA47*. The Mann-Whitney test and Kruskal-Wallis test with Dunn's posttest show that the WT and complemented

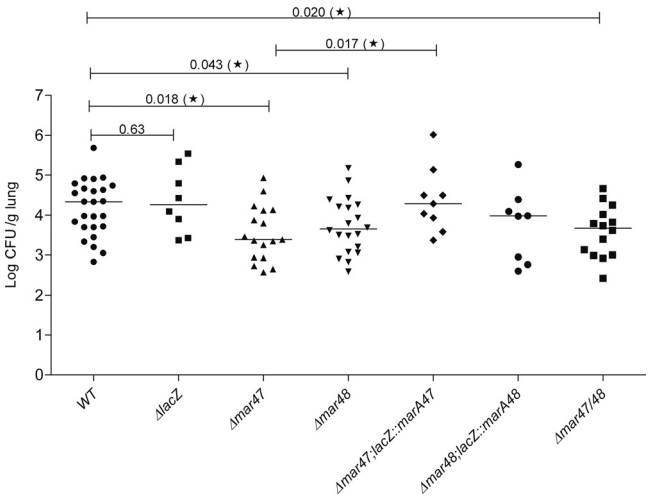

FIG. 4. Lung colonization after intranasal infection. Female BALB/c mice were intranasally infected with 2×10^5 CFU of each strain: WT, ILKIM1 (Δ*marA47*), ILKM9 (Δ*marA47*), ILKIM11 (Δ*marA47* Δ*marA48*), ILKIM66 (Δ*lacZ*), ILKIM128 (Δ*marA48* *lacZ*::*marA48*), and ILKIM131 (Δ*marA47* *lacZ*::*marA47*). After 5 days, mice were euthanized, and the lungs were homogenized, serially diluted, and plated. Viable CFU were counted and divided by the mass of lung tissue to give the log CFU/g lung. Each dot in the graph shows the result for one mouse. The horizontal bar denotes the median. The *P* values between two strains are given above the horizontal bars as determined by the Mann-Whitney nonparametric test. Asterisks indicate significant differences ($P < 0.05$).

strains are not significantly different. While the complemented *marA48* strain (ILKIM128) showed increased numbers of colonies in the lungs, it was not significantly different from the *marA48* deletion strain. This could be due to the location of the complemented gene in the *lacZ* locus, which somehow prevents its functioning like the WT in the mouse. This explanation seems feasible, since *marA48* (also called *rob*) has recently been shown to be upregulated in the first 12 to 48 h in the lungs and liver during a pneumonic *Y. pestis* mouse infection (19). These results suggest that loss of *marA48* could result in a less virulent organism and therefore support our finding that loss of *marA48* makes the strain less able to colonize lungs. That the deletion of both genes together is not significantly different from the deletion of either gene alone suggests that their functions are not additive, unlike the effect of the double deletion in comparison with the single deletions in the antibiotic susceptibility tests (see above and Fig. 2). As described above for the antibiotic susceptibility study, no difference was seen in growth rates in single-strain culture growth curves or in coculture studies (data not shown). Therefore, the difference in lung colonization is not caused by a difference in growth rates between the strains.

It has also been shown that the importance of genes in infections depends on the route of infection. For example, *Y. pestis* deleted of *rovA*, a MarR/SlyA family transcriptional regulator, is attenuated in dissemination of bacteria via the subcutaneous route but not via the intranasal route (5). However, as KIM 1001 Δ*pgm* is highly attenuated via the subcutaneous route, requiring $>10^7$ CFU to cause disease (42), we were unable to mimic a bubonic plague infection to determine the importance of *marA47* and *marA48* via this route. Further experiments are planned in which these genes will be deleted in a fully virulent strain and thus their importance in bubonic plague can also be tested.

Plague patients can be successfully treated with antibiotics. It is therefore of great concern that two strains from two districts in Madagascar were found to be resistant to antibiotics favored in treatment and prophylaxis (10). Should these types of strains become more widespread or be used as bioweapons, treatment of patients, especially in large numbers, would be difficult. We have shown that two regulatory proteins are involved in antibiotic resistance and virulence. MarA47 is involved with susceptibility to several different classes of antibiotics, and our results expand on overexpression data previously reported (41). MarA48 appears to affect fluoroquinolone and aminoglycoside (when overexpressed) susceptibility (Fig. 2 and 3) and appears to enhance the function of MarA47 in antibiotic susceptibility. Both genes also affected colonization of mouse lungs (Fig. 4).

This finding suggests that different pathways are involved and that these two genes represent potential treatment targets by which antibiotic susceptibility and virulence could be addressed together. Studies of *E. coli* and *Salmonella* spp. show that the regulons of MarA, SoxS, and Rob are large, and other studies (2, 3, 26, 27) indicate that there is an intricate network of response mechanisms by which these transcriptional regulators are controlled and by which they control downstream proteins, including other transcriptional regulators (7, 15, 29, 33–35). Numerous proteins are involved in colonization during disease, so MarA47 and MarA48 could have a number of different roles. For example, MarA, SoxS, and Rob in *E. coli* are all known to regulate the transcription of the drug efflux pump AcrAB-TolC (a major mechanism of antibiotic resistance) and the transcription of outer membrane porins such as OmpF (2, 3, 26). MarA47 controls expression of *acrAB* in *Y. pestis* to make the bacteria less susceptible to antibiotics (41) and likely to antibacterial mammalian compounds such as cationic antimicrobial peptides. The loss of *marA47* would then lead to a decrease of the levels of *acrAB*, making the bacteria more susceptible to host defense mechanisms. Ongoing and future work will determine the MarA47 and MarA48 regulons and address how they produce antibiotic resistance and virulence.

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